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(54) Title: A HIGH-AFFINITY HUMAN GLUTAMATE/ASPARTATE UPTAKE SYSTEM

(57) Abstract

Human neurotumor cells capable of being grown in culture and having a specific high-affinity glutamate/aspartate transport system are provided. Further, is a clonal derivation cell line SH-EP having a flat epithelial morphology and has a specific high-affinity Glutamate/Aspartate transport system. Methods are provided for deriving the SH-EP cells and for using SH-EP cells to detect agonists and antagonists for a glutamate/aspartate transport system. The transport and pharmacological characteristics of this cell line are described.

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A HIGH-AFFINITY HUMAN GLUTAMATE/ASPARTATE UPTAKE SYSTEM

Field of the Invention

The present invention relates generally to cells having a high-affinity glutamate/aspartate uptake transport system, and the use of these cells for identifying agonists and antagonists directed at these transport systems.

Background of the Invention

Nerve cells ("Neurons") predominantly utilize chemical messengers known as "neurotransmitters" (or "transmitters") to communicate with each other. This process of neuronal communication is referred to as "chemical neurotransmission" and includes four sequential components: transmitter synthesis/storage, transmitter release, transmitter reception and transmitter inactivation. To begin this process of neurotransmission, each neuron synthesizes and/or stores appropriate transmitters for the purpose of conveying discrete information to other neurons. With appropriate stimulus, a pre-synaptic neuron releases its content of neurotransmitters into the "synapse," or extracellular space outside of the transmitter-releasing neuron that apposes the transmitter-receiving (or post-synaptic) neuron. The transmitters then diffuse until they contact, or bind to, appropriate receptors within this synaptic space.

While the released neurotransmitters are searching for receptors in the synapse, they are concomitantly susceptible to "inactivation" and thereby prevented from being recognized by receptors. This inactivation

process generally occurs via one of two mechanisms: (1) enzymes present in the synapse that alter the neurotransmitters into chemical forms not recognized by corresponding receptors; or (2) transport (or uptake) systems on the pre-synaptic neuronal surface that re-internalizes the released neurotransmitters, thereby effectively decreasing the concentration of available transmitters to activate receptors. The former mechanism is the principal means whereby acetylcholine is inactivated in a chemical synapse; the latter mechanism is believed to be the principal means whereby amino acid (e.g., glutamate, aspartate, etc.) and biogenic amine (e.g., serotonin, dopamine, etc.) neurotransmitters are inactivated during neurotransmission. Akin to receptor systems, wherein each receptor preferentially recognizes its corresponding neurotransmitter, transport systems are also specific in that each recognizes and transports only its corresponding neurotransmitter. In addition to their integral role(s) in regulating neurotransmission, these transport systems are of clinical and pharmaceutical interest because some of their blockers (or "inhibitors") are the only effective means of treating certain diseases. This accounts for the intense academic as well as pharmaceutical interest in neurotransmitter transport mechanisms.

There have been reports on the use of cell lines and primary cell cultures derived from the brain for analysis of a glutamate/aspartate transport system. For example, Nicklas and Browning, *J. of Neurochem.* 41:179-187 (1983); Deas and Erecinska, *Brain Res.* 483:84-90 (1989); Cho and Bannai, *J. of Neurochem.* 55:2091-2097 (1990); Schousboe, et al, *J. of Neurochem.* 29:999-1005 (1977); Schousboe and Divac, *Brain Res.* 17:407-409 (1979); Stallcup, et al, *J. of Neurochem.* 32:57-65 (1979); Hansson, et al, *Neurochem. Res.* 10:1335-1341 (1985); Erecinska, et al, *Brain Res.* 369:203-214 (1986); Yu, et al, *J. of Neurosci. Res.* 17:424-427 (1987); Barbour, et al, *Nature* 335:433-435 (1988); Hansson and Ronnback, *Life Sci.* 44:27-34 (1989); and Nilsson, et al, *Neuropharmacology* 28:1415-1418 (1989), show studies of glutamate/aspartate transport systems using

primary cultures of neurons, glial cells or glioma cells. These studies have focused on cells derived from mice and rats, with the exception of Barbour which used salamander retina. None of these studies discuss the isolation of a glutamate/aspartate uptake system from human neurological cells. The present invention is the first disclosure of a high affinity glutamate/aspartate uptake system from human neurotumor cells.

Summary of the Invention

An object of the present invention is the isolation, characterization and identification of a specific high-affinity glutamate/aspartate transport system.

An additional object of the present invention is a method of detecting agonists and antagonists for the high-affinity glutamate/aspartate transport system.

Thus in accomplishing the foregoing objects, there is provided in accordance with one aspect of the present invention as a composition of matter, a cell line characterized as being a human neurotumor cell capable of growth in culture, having a flat epithelial morphology, being a clonal derivative of SK-N-SH cell line, and having a specific high-affinity glutamate/aspartate transport system.

The transport system is capable of transporting specifically L-aspartate, D-aspartate and L-glutamate. The system is sodium dependent and can be inhibited by cobalt, nickel, cadmium ions. Other inhibitors of the system include threo-3-hydroxy-D,L-aspartate, L-cysteine sulfinate and L-cysteate.

A further aspect of the present invention is a method for screening for agonists or antagonists for the glutamate/aspartate transport system. This method includes contacting the SH-EP or SK-N-SH cells with a compound to be tested and measuring the intracellular presence of said compound.

Another aspect of the present invention is a method of isolating a SH-EP cell line, including dilating cultured SK-N-SH cells into culture plated wells; repopulating the plate well; retaining single cell colonies and repeating the diluting, repopulating and retaining steps at least once.

Other and further objects, features and advantages shall be apparent and eventually more readily understood from a reading of the following specification and by reference to the accompanying drawings from and apart thereof, where examples of the presently preferred embodiments of the invention are given for the purposes of disclosure.

Description of the Drawings

Figure 1 shows the kinetic profiles of the glutamate/aspartate uptake in SH-EP cells. Figure 1A shows the uptake for D- and L-^{3}H-Asp and Figure 1B shows the uptake for L-^{3}H-Glu.

Figure 2 shows the specificity of D-^{3}H-Asp uptake in SH-EP cells.

Figure 3 shows the effect of inhibitors on the profile of D-^{3}H-Asp uptake in SH-EP cells.

Detailed Description of Invention

It will be readily apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

One embodiment of the present invention as a composition of matter is a cell line characterized as being a human neurotumor cell, being capable of growth in culture, having flat epithelial morphology, being a clonal derivative of a SK-N-SH cell line and having a specific high-affinity glutamate/aspartate transport system.

The SH-EP cells are a clonal derivative of the human neuroblastoma SK-N-SH cells. The clonal derivative was prepared by the following steps: Cultured SK-N-SH cells were released by trypsinization

and serially diluted for plating into 96-well culture plates. When the plated cells start to repopulate the plate wells, only those containing single cell-colonies were retained for further characterization. This process of cloning by limiting dilution generates clonal cell lines with distinct morphologies; SH-EP cells arising from this process possess a flat morphology, strong substrate adhesiveness and the ability to grow as a monolayer. This cloning process is repeated at least once to ensure the morphological homogeneity and stability of the attained SH-EP cell line.

The SK-N-SH and SH-EP cell lines were further characterized by their high-affinity transport system. Both SK-N-SH and the isolated SH-EP cell lines of the present invention have a high-affinity glutamate/aspartate transport system. The system is characterized by its affinity to the ligands L-aspartate, D-aspartate and L-glutamate. The ligand affinity is L-aspartate > D-aspartate > and L-glutamate.

Another embodiment of the present invention is a procedure for screening for agonists and antagonists for the high-affinity glutamate/aspartate transport system. This procedure provides a method for analyzing novel pharmacological agents and for further understanding the molecular mechanisms underlying this neurotransmitter transport system. The procedure for screening for agonists and antagonists comprises the steps of contacting a human neurotumor cell with a compound to be tested, wherein said human neurotumor cell is capable of growth in culture and has a specific high-affinity glutamate/aspartate transport system, and measuring the intracellular concentration of the compound. In this method, SH-EP or SK-N-SH cells were used, however, SH-EP cells were used in the preferred mode. One skilled in the art will readily recognize that there are a variety of methods to determine the intracellular concentration of a compound. This can be achieved by measuring the concentration of the compound with standard chemical, electrochemical and biochemical methods. This could include using

labelled and unlabelled compounds, antibodies or inhibition assays. In the preferred method the test compound is radiolabelled.

SK-N-SH and SH-EP cells were cultured in Modified Eagle's Medium supplemented with 10% fetal calf serum (GIBCO), released from the culture vessel by trypsinization and plated into 96-well plates for neurotransmitter transport assays. This assay involves the following procedure: Culture medium is first removed from each plate well. The confluent cell monolayer remaining in the well is then rinsed twice with Ringer's buffer (128 mM NaCl, 5.2 mM KCl, 2.1 mM $CaCl_2$, 2.9 mM $MgSO_4$, 5 mM glucose, 10 mM HEPES; bubbled with oxygen gas and pH adjusted to 7.4 prior to use) and incubated with 50 ml of gas and pH adjusted to 7.4 prior to use) and incubated with 50 ml of Ringer's buffer plus D-[^{3}H]Asp. The transport incubation is conducted at physiological temperature (37°C) for up to 40 minutes and terminated by removal of excess D-[^{3}H]Asp from the well. After three rinses with Ringer's buffer, the cell monolayer is lysed into solution 1% SDS and transferred into a scintillation vial for liquid scintillation spectrometry to quantitate the level of D-[^{3}H]Asp transported by the cells.

The following examples are offered by way of illustration and are not intended to limit the invention in any manner. In examples, all percentages are by weight for solids and by volume for liquids and all temperatures are in degrees Celsius unless otherwise noted.

Example 1

Kinetic Profiles of SH-EP Cells

Double reciprocal plots (1/v versus 1/s) of initial uptake velocities for both D- and L-^{3}H-Asp and L-^{3}H-Glu using ligand concentrations ranging from 1-50μM were determined. Specific velocities of initial uptake were determined as the differences in velocity values observed at 37°C and at 0°C (on ice). Each of the uptake measurements were taken after ten minutes incubation. The results of these kinetic profiles are plotted

in Figures 1A and 1B. Each plotted point represents the mean ± standard deviation for 6 assays. The SH-EP cell line accumulated exogenous ligand in a temperature dependent manner. Further, the ligand accumulation increased as a function of time. The resulting velocity profiles were all saturable and when transformed into Lineweaver-Burk plots (Figure 1) yielded the following transport parameters for each ^{3}H-ligand: (1) D-^{3}H-Asp: K_m = 6.9 ± 2.1μM, V_{max} = 47.2 ± 13.0 pmol/mg/min; (2) L-^{3}H-Asp: K_m = 4.8 ± 0.7μM, V_{max} = 39.6 ± 3.9 pmol/mg/min; (3) L-^{3}H-Glu: K_m = 22.5 ± 8.3μM, V_{max} = 1.0 ± 0.3 nmol/mg/min.

The transport K_m for these ^{3}H ligands are comparable to the transport affinities of other Glutamate/Aspartate uptake system. The transport V_{max} values for both stereoisomers of ^{3}H-Asp were in close agreement with each other, and supports the notion that both D- and L-aspartate are ligands for a common transport mechanism. By comparison, however, the transport V_{max} of L-^{3}H-Glu was significantly higher than that of D- and L-^{3}H-Asp. This discrepancy in V_{max} (1) may reflect a real kinetic distinction on the part of the high-affinity Glutamate/Aspartate transport system; or (2) may reflect an additional transport mechanism for glutamate exists within this concentration range of ^{3}H ligand.

Example 2

Ligand Specificity

To assess the ligand specificity of the high-affinity glutamate/aspartate transport system of SH-EP cells, several amino acids, amino acid neurotransmitters and amino acid analogues were used as competing ligands. In the present example, 1 μM D-^{3}H-Asp was incubated with the SH-EP cells at 37°C for 40 minutes. Each competing ligand was at a final concentration of 100μM. The results using the following competing ligands L-Aspartate (L-Asp), D-Aspartate (D-Asp), L-Glutamate (L-Glu), D-Glutamate (D-Glu), L-Glutamine (L-Glu), α-ketoglutarate

(a-KG), γ-aminobuteric acid (GABA), taurine (Tau), L-tryptophan (L-Try), L-threonine (L-Thr), L-cystine, glycine (Gly), L-lysine (L-Lys) and L-valine (L-Val) are shown in Figure 1. Each bar in Figure 1 represents the mean ± standard deviation of 6 assays expressed as a percent of control uptake level. Of the ligands tested, only D- and L-Aspartate and L-Glutamate exhibited significant inhibition; strongly supporting the specificity of the transport system to the transport ligands. From the inhibition results and the apparent transport affinities, it is obvious that the rank order of ligand affinities for this system is L-Aspartate > D-Aspartate > L-Glutamate > D-Glutamate.

Example 3

Inhibition Profile of the

Glutamate/Aspartate Uptake System

The pharmacological profile of the uptake system was examined using known inhibitors of previously existing high-affinity Glutamate/Aspartate uptake systems and excitatory amino acid receptor ligands. SH-EP cells were incubated at 37°C for 40 minutes with 1μM D-^{3}H-Asp in the presence or absence of inhibitor. The inhibitor was at 100μM final concentration. All data were normalized by protein content and expressed as a percentage of control value.

Each bar represents the mean ± standard deviation of 6 assays. The uptake inhibitors included threo-3-hydroxy-D,L-aspartate (THAA), L-cysteine sulfinate (L-CSA) and L-cysteate (L-CA). As seen in Figure 3 all three of these inhibitors were potent inhibitors of the Glutamate/Aspartate transport system. Ligands specific for various Glutamate/Aspartate receptor subtypes were also tested as inhibitors. These test ligands included N-methyl-D-aspartate (NMDA), quisqualate (QA), kainate (KA), dihydroxykainate (DHKA), L-Glutamate diethylester (DEEG), D,L-2-amino-3-phosphonopropionate (APPA), D-2-aminoacipate

(AAA) and L-homocysteate (L-HCA). As seen in Figure 3, none of these exhibited significant inhibition of this uptake system.

Example 4

Ion Dependence of the Glutamate/Aspartate Transport System in SH-EP Cells

The ion dependence of the glutamate/aspartate high-affinity transport system in SH-EP cells was examined by substituting individual ion species in the assay. Ringer's buffer with ionic alternatives was used. In the present example, Na^+ was substituted by Li^+; Cl^- was substituted by isethionate; Ca^{++} was substituted by Mg^{++}. Each resulting Ringer's composition was used to test for its affect on D-^{3}H-Asp uptake by SH-EP cells. The uptake assay contains 1μM, D-^{3}H-Asp and was performed at 37°C for 40 minutes. All data were normalized by protein content and expressed as percent of control uptake levels (mean ± standard deviation). The results of this analysis are shown in Table 1.

TABLE 1

Ringer's Buffer	% Control Uptake
Na-free	1.5 ± 0.3
Cl-free	79.0 ± 9.1
Ca-free	81.2 ± 7.6
Co/Ca	5.7 ± 0.3
Ni/Ca	0.8 ± 0.1
Cd/Ca	43.7 ± 1.7

The glutamate/aspartate high-affinity uptake system of the present invention is highly Na^+ dependent but only shows limited dependence on Cl^- and Ca^{++}. It is noteworthy that when Ca^{++} was replaced by Co^{++}, Ni^{++} or Cd^{++} significantly more uptake inhibition was observed than when Ca^{++} was absent. Thus, the divalent metal ions were exerting a direct

inhibitory effect upon the D-^{3}H-Asp. The rank order of inhibitory potencies for the divalent ions are Ni^{++} >, Co^{++} >, Cd^{++}.

Example 5

Glutamate/Aspartate Receptor in SK-N-SH Cells

The human neuroblastoma SK-N-SH cell line, progenitor of the SH-EP cell line, was also characterized for high-affinity glutamate/aspartate uptake. By using D-[^{3}H]Asp as transport ligand. The SK-N-SH cell line possesses a glutamate/aspartate transport system with the same properties as the transport system described for the SH-EP cell line: (1) the transport system has a kinetically saturable profile characterized by a transport K_m of between about 5 and 10 μM, thereby indicating that the transport system is high-affinity; (2) the same series of competing ligands were used for SK-N-SH as described above for SH-EP cells, and only D-Aspartate, L-Aspartate and L-Glutamate exhibited significant inhibition. The rank order of ligand affinities was the same. (3) several uptake inhibitors, including THAA, L-CSA and L-CA, effectively inhibited this system; (4) ligands specific for various glutamate/aspartate receptor subtypes were not effective inhibitors of this transport system; (5) this system is highly Na^+-dependent, and only partly dependent on Cl^- and Ca^{++}; (6) inhibitory effects were observed for Ni^{++}, Co^{++} and Cd^{++}. These transport properties are exactly those discerned for the glutamate/aspartate transport by the SH-EP cell line. Since the SH-EP cell line is a clonal descendent of the SK-N-SH cell line, it is concluded that both cell lines manifest the same high-affinity glutamate/aspartate transport system.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The cell lines and methods, procedures and techniques described herein are presently representative of the preferred embodiments and are intended to be

exemplary and are not intended as limitations on the scope. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention and are defined by the scope of the appended claims.

WHAT IS CLAIMED IS:

1. As a composition of matter a SH-EP cell line characterized as:

being a human neurotumor cell;

being capable of growth in culture;

having a flat epithelial morphology;

being a clonal derivative of a SK-N-SH cell line; and

having a specific high-affinity Glutamate/Aspartate transport system.

2. The cell line of claim 1, wherein the high-affinity transport system is specific for ligands selected from the group consisting of L-aspartate, D-aspartate and L-glutamate.

3. The cell line of claim 1, wherein the order of ligand affinity is L-aspartate > D-aspartate > L-glutamate.

4. The cell line of claim 1, wherein said transport system is highly Na^+ dependent.

5. The cell line of claim 1, wherein the transport system has limited Ca^{++} or Cl^- dependence.

6. The cell line of claim 1, wherein Co^{++}, Ni^{++} or Cd^{++} inhibit ligand uptake by the transport system.

7. The cell line of claim 1, wherein said transport system is inhibited by the group consisting of threo-3-hydroxy-D,L-aspartate, L-cysteine sulfinate and L-cysteate.

8. The cell line of claim 1, wherein the transport system is not significantly inhibited by the receptor ligands of the group consisting of N-methyl-D-aspartate, quisqualate, kainate, dihydroxykainate, L-glutamate diethylester, D,L-2-amino-3-phosphonopropionate, D-2-aminoadipate and L-homocysteate.

9. A method of isolating the SH-EP cell line of claim 1 comprising the steps of:

diluting cultured SK-N-SH cells into culture plate wells;

repopulating the plate wells;

retaining single cell colonies; and

repeating the diluting, repopulating and retaining steps at least once.

10. A method for screening for agonists or antagonists for a glutamate/aspartate high-affinity transport system, comprising the steps of:

contacting a human neurotumor cell with a compound to be tested, wherein said human neurotumor cell is capable of growth in culture and has the specific high-affinity glutamate/aspartate transport system; and

measuring the intracellular concentration of said compound.

11. The method of claim 10, wherein said compound is radiolabelled.

12. The method of claim 10, wherein said assay further includes a competitive inhibitor and either the inhibitor or test compound is measured.

13. The method of claim 10, wherein said cell is from the SH-EP cell line.

14. The method of claim 10 wherein said cell is from the SK-N-SH cell line.

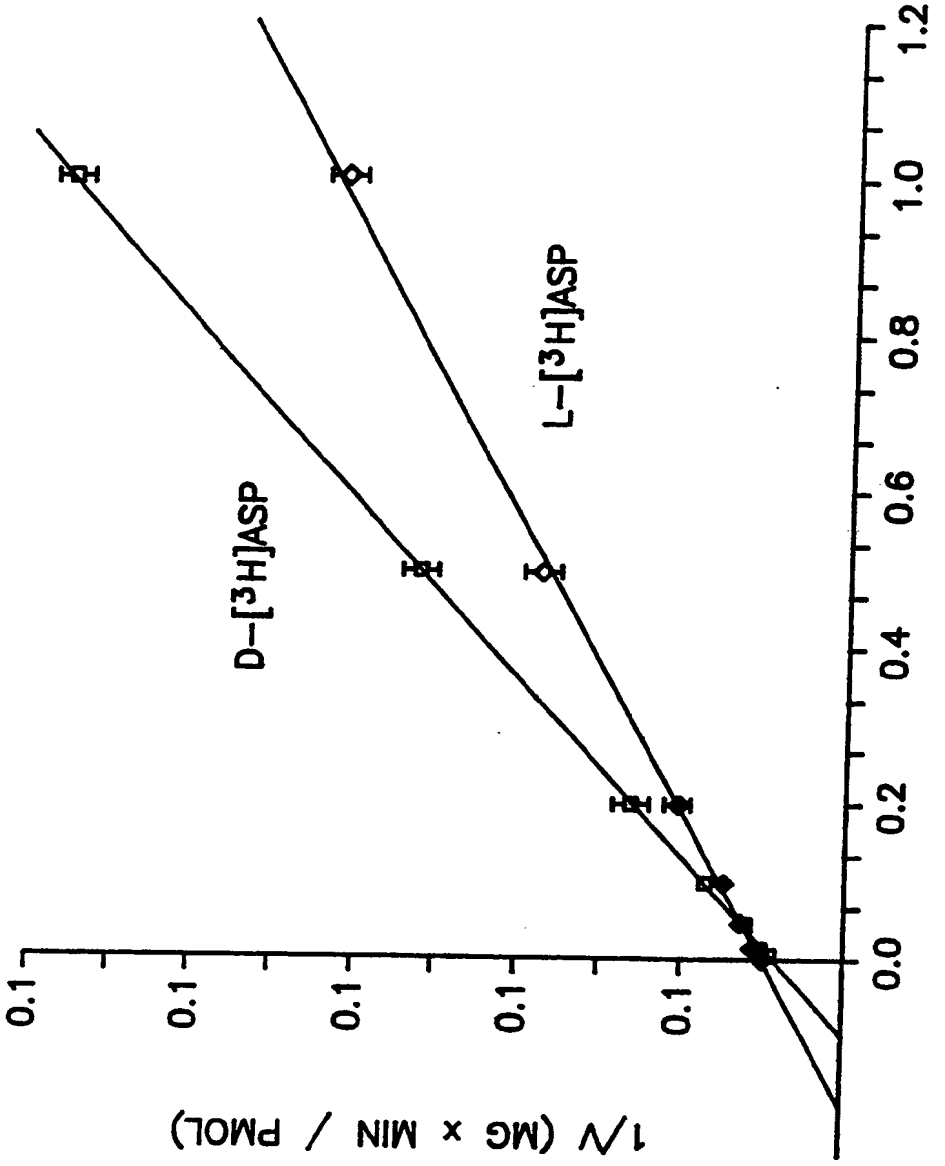

FIG. 1A

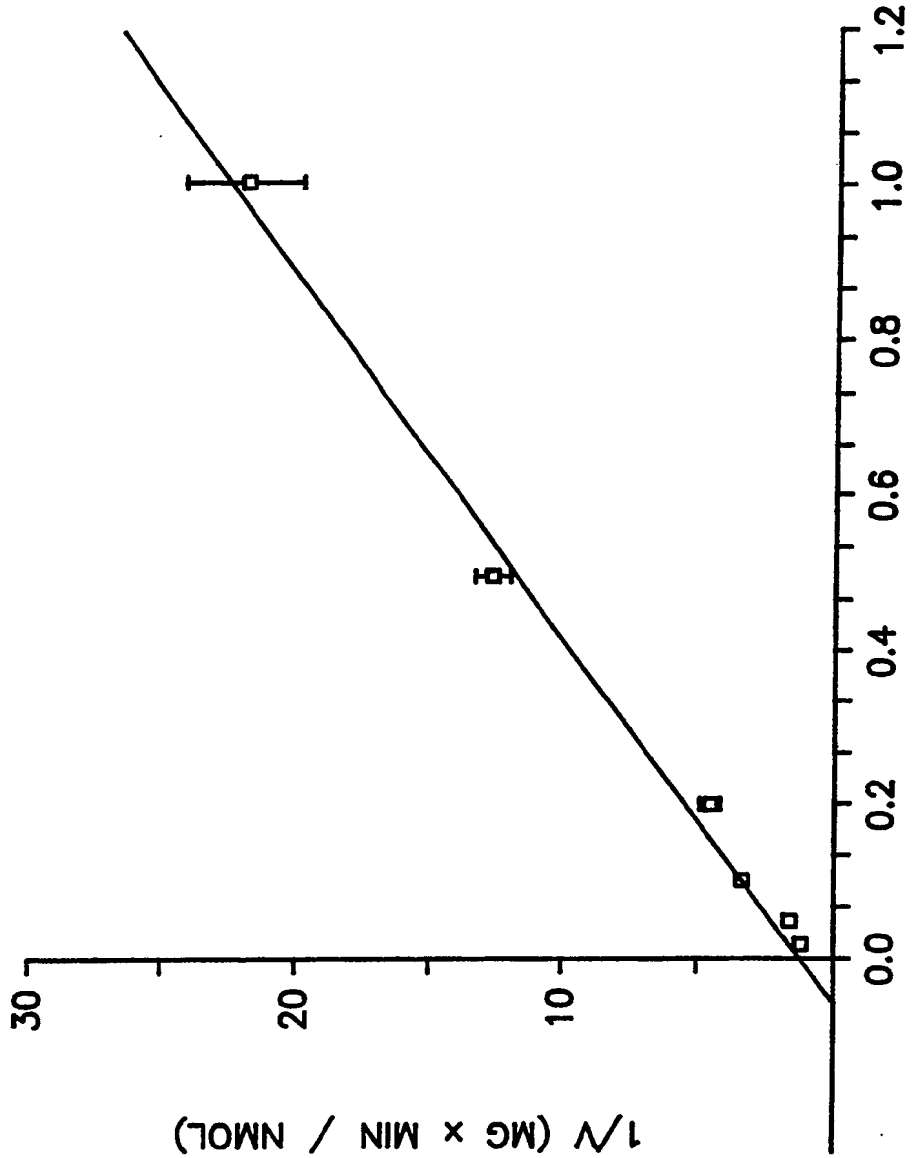

FIG. 1B

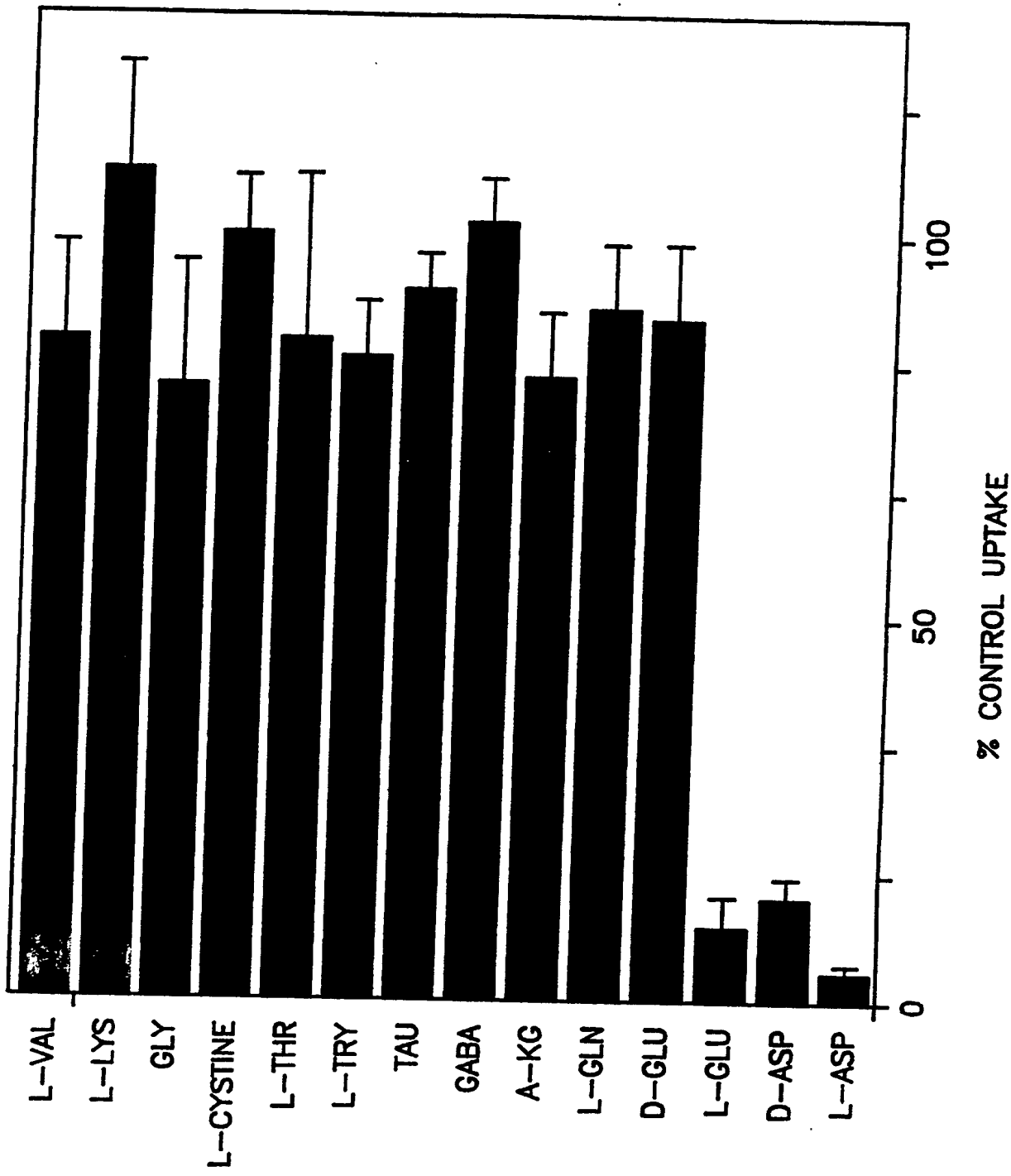

FIG. 2

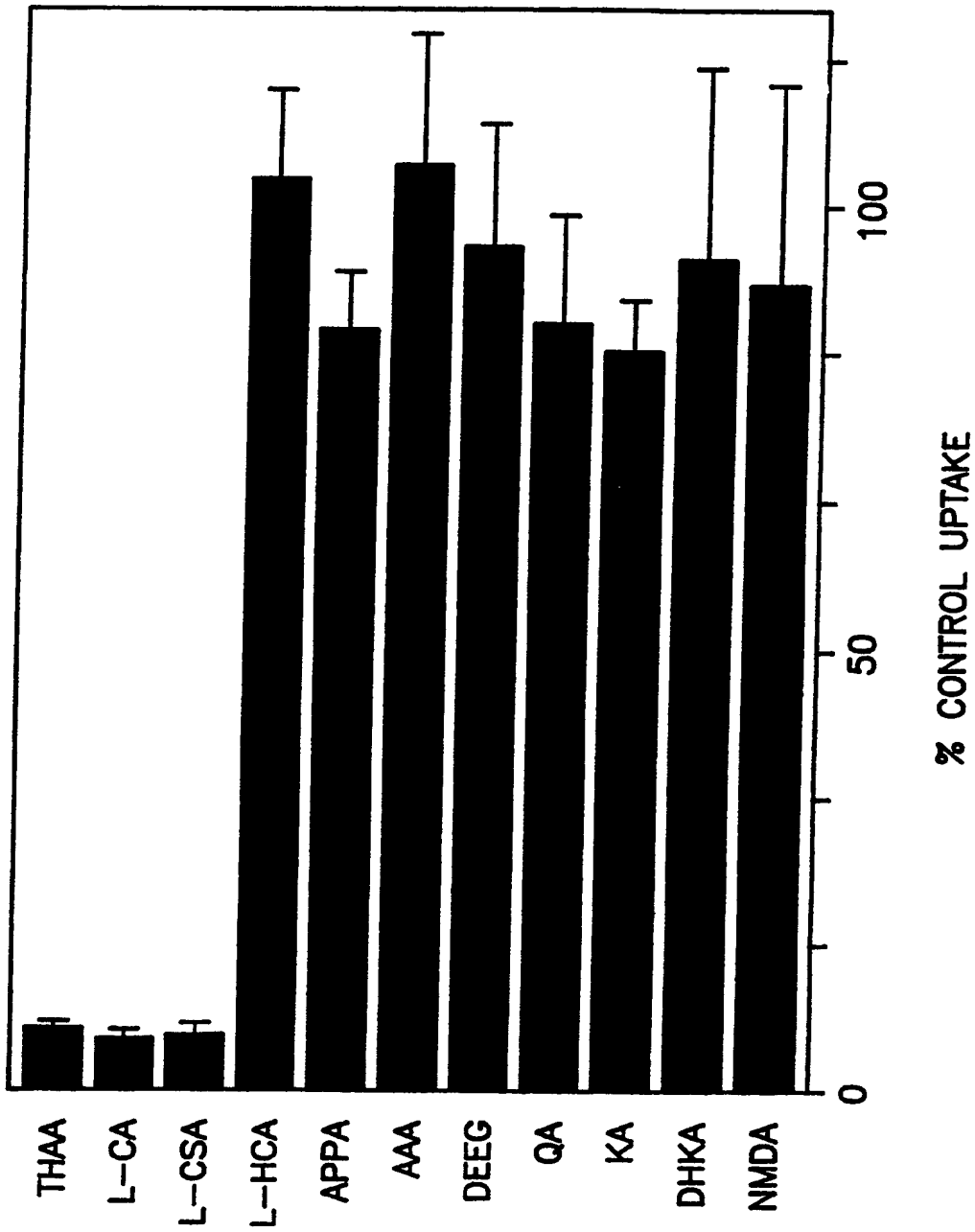

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 1/00, 5/00; C12Q 1/00, 1/02
US CL :435/240.2, 240.21, 948, 4, 29
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.2, 240.21, 948, 4, 29

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	"ATCC Catalogue of Cell Lines & Hybridomas", 6th Edition, published in 1988, pages 196-197 and 241-242, see entire document.	1-8
Y	Journal of the National Cancer Institute, Vol. 71, No. 4, issued October 1983, Ross et al, "Coordinate Morphological and Biochemical Interconversion of HUman Neuroblastoma Cells", pages 741-747, see entire document.	1-9
X – Y	Cancer Research, Vol. 47, issued 01 October 1987, Sadee et al, "Expression of Neurotransmitter Receptors and myc Protoncogenes in Subclones of a Human Neuroblastoma Cell Line", Pages 5207-5212, see entire document.	1-14 ---- 1-14
Y	Journal of Neurochemistry, Vol. 19, issued 1972, Balcar et al, "The Structural Specifcity of the High Uptake of L-Glutamate and L-Aspartate by Rat Brain Slices", pages 2657-2666, see entire document.	1-8

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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INTERNATIONAL SEARCH REPORT

International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, Vol. 335, issued 29 September 1988, Barbour et al, "Electrogenic Glutamate Uptake in Glial Cells is Activated by Intracellular Potassium", pages 433-435, see entire document.	1-8
Y	Cancer Research, Vol. 33, issued November 1973, Biedler et al, "Morphology and Growth, Tumorigenicity, and Cytogenetics of Human Neuroblastoma Cells in Continuous Culture", pages 2643-2652, see entire document.	1-8
Y	Neurochemistry International, Vol. 10, No. 3, issued 1987, Cowburn et al, "A Comparison of High Affinity Synaptosomal Uptake of D-Aspartate in Rat and Human Brain", pages 339-346, see entire document.	1-8
Y	Brain Research, Vol. 490, issued 1989, Dodd et al, "Uptake of GABA and L-Glutamic acid by Synaptosomes from Postmortem Human Cerebral Cortex: Multiple Sites, Sodium dependence and Effect of Tissue Preparation", pages 320-331, see entire document.	1-8
Y	Journal of Neurochemistry, Vol. 53, No. 3, issued 1989, Hollmann et al, "Glutamate Transport and Not Glutamate Receptor Binding is Stimulated by Gangliosides in a Ca^{2+}-Dependent Manner in Rat Brain Synaptic Plasma Membranes", pages 716-723, see entire document.	1-8
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Y	Biochemical Pharmacology, Vol. 35, No. 6, issued 1986, Anand et al,"Effects of Kainic Acid Analogues on the HIgh Affinity Uptake of D-[^{3}H]Aspartate into Rat Cerebrallar Homogenates", pages 1055-1057, see entire document.	10-14
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